PROBES OF DNA STRUCTURE AND INTERACTIONS

EFFECTS OF COPPER II ON ULTRAVIOLET-INDUCED PYRIMIDINE DIMER FORMATION

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ABSTRACT Cu(II) affects the yield of cyclobutyl dimers induced in DNA by 254 nm radiation. The effects are a function of *r*, the ratio of Cu(II) to DNA phosphate, and of the ultraviolet (UV) fluence; they seem to reflect two types of copper complexes with DNA. The first probably involves "exterior" binding to the bases of native DNA and increases $\widehat{TT}$ formation (without affecting $\widehat{UT}$ yield) by raising the energy levels of bases other than thymine. The second seems to occur only at high ratios (*rs*) and only after the structure has been opened locally by UV radiation; it involves "interior" binding of Cu(II) to the bases. This complex tends to decrease dimer yield by holding the bases apart and/or by lowering the energy levels of bases other than thymine. These results illustrate the potential use of DNA photoproducts and ligands to probe the structure and interactions of DNA in vitro and perhaps also in vivo.

INTRODUCTION

Cyclobutane-type pyrimidine dimers, produced in DNA by ultraviolet (UV) radiation (220–300 nm) are of chemical interest because of their lethal and mutagenic effects. (See, for example, review by Setlow, 1966.) They can also serve as photochemical probes of DNA structure and interactions (Stafford and Donnellan, 1968; Longworth, 1968; Sutherland and Sutherland, 1969 *a*; 1969 *b*). Introduction of energy traps into the DNA structure can alter photoproduct yield (Sutherland and Sutherland, 1969 *a*, 1969 *b*). Longworth and Rahn (1967) have shown that lowering the energy levels of a residue in poly-L-tyrosine by ionization produces a trap for excitations from neighboring residues. Alteration of base levels in DNA by ionization produced by pH changes is not possible without seriously perturbing the helical structure. However, certain metals which bind to the bases of DNA might perturb

their energy levels without affecting the helical structure. Thus, we thought that examination of photoproducts induced in DNA in the presence of metal ions could provide a probe for studying DNA-metal interactions.

We have studied the effect of Cu(II) on the production of cyclobutyl pyrimidine dimers both as a function of the ratio of Cu(II) to DNA phosphate, *r*, and as a function of the incident UV fluence. We find that Cu(II) forms two types of complexes with DNA bases. The first is formed with native DNA and increases thymine-thymine dimer yield without affecting cytosine-thymine dimer yield. This effect reflects an increase in the energy levels of other bases relative to thymine. The second is formed only for high *rs* after the structure has been opened by UV. This complex decreases dimer yield by holding the bases apart and/or by lowering the energy levels of other bases with respect to thymine.

MATERIALS AND METHODS

Assay of Radioactive DNA's for Pyrimidine Dimers

Methods of labeling DNA and production of, assay for, and identification of pyrimidine dimers have been described previously (Sutherland and Sutherland, 1969 *a*; Sutherland et al., 1968). In addition to the DNA described previously (*Escherichia coli*, thymidine-methyl-^{3}H, 10^4 cpm/μg) (Sutherland and Sutherland, 1969 *a*), we also used the following *E. coli* DNA's, the gift of W. L. Carrier and R. B. Setlow: cytidine-5-^{3}H, 1.4×10^4 cpm/μg; thymidine-2-^{14}C, 2.7×10^4 cpm/μg; and thymidine-methyl-^{3}H, 1.6×10^5 cpm/μg. All DNA's were purified by Marmur's (1961) method.

Briefly, the DNA's were irradiated with stirring (1.0 cm path, 254 nm, fluences corrected for absorption by the DNA by Morowitz' [1950] corrections) in 0.005 M NaCl, pH 5.5 in the presence of Cu(II). Incident UV fluxes were monitored with a Jagger (1961) meter.

The samples were hydrolyzed in formic acid, chromatographed on cellulose thin layers or on Whatman No. 1 paper in butanol:acetic acid:water (40:6:15) (Smith, 1963), counted in a scintillation counter and analyzed for thymine and thymine-containing dimers, or cytosine and cytosine-containing dimers (deaminated during hydrolysis to uracil-containing dimers). The dimers thymine-thymine, uracil-thymine and uracil-uracil will be abbreviated as $\widehat{TT}$, $\widehat{UT}$, and $\widehat{UU}$, respectively.

Preparation of Cu(II)-DNA Solutions

Extensively-purified $CuCl_2$ was obtained from the Department of Organic Chemistry of the Walter Reed Army Institute. Solutions were prepared by dissolving the DNA's in 0.005 M NaCl, pH 5.5 and then adding Cu(II) from a stock solution of 10^{-3} M Cu(II) in 0.005 M NaCl, pH 5.5, to the desired concentration. NaCl was used to prevent precipitation of Cu(II) noted with some salts and buffers.

Spectrophotometry

Nonradioactive, purified, *E. coli* DNA in 0.005 M NaCl, pH 5.5 was exposed to 254 nm radiation in the presence of Cu(II); then absorption spectra were taken with a Cary 14 (Applied Physics Corp., Monrovia, Calif.).

RESULTS AND DISCUSSION

For fluences of 4×10^3 Jm^{-2}, increasing ratios of Cu(II):DNA (phosphate), r, monotonically increase the yield of thymine-thymine dimers without affecting uracil-thymine dimer yield (Fig. 1). We identified the photoproducts formed in the presence of Cu(II) as $\widehat{TT}$ and $\widehat{UT}$ by (*a*) their composition, (*b*) their chromatographic mobility, (*c*) cochromatography with $\widehat{TT}$ and $\widehat{UT}$ produced in the absence of Cu(II), and (*d*) their reversibility to the monomers by 254 nm irradiation of the isolated photoproducts in solution.

We can exclude four possible mechanisms of increasing dimer yield. First, since the molar extinction coefficient of Cu(II) at 254 nm is very small (<50), transfer of energy absorbed by Cu(II) to the DNA cannot account for the increased dimer yield. Second, since dimer yield for a given UV fluence is higher for denatured than for native DNA, and under certain conditions, Cu(II) can destabilize DNA, the increased dimer yield might result from denaturation produced cooperatively by UV and Cu(II). Several lines of evidence argue against this. (*a*) Denaturation should affect $\widehat{TT}$ and $\widehat{UT}$ yields similarly; however, Fig. 1 shows that $\widehat{TT}$ increases while $\widehat{UT}$ does not. (*b*) Complete denaturation would be required to account for the magnitude of the increase in $\widehat{TT}$.[1] However, Fig. 2 shows that at $r = 2$, there is

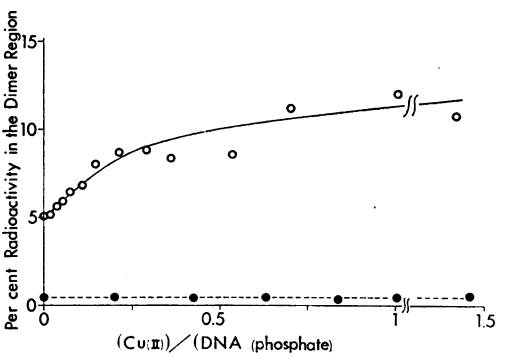

FIGURE 1 A typical experiment showing thymine-thymine dimer (○) and uracil-thymine dimer (●) yields as a function of the Cu(II) to DNA phosphate ratio at 4×10^3 Jm^{-2}. $\widehat{TT}$ increases with increasing r, but $\widehat{UT}$ does not. Thymidine-^{3}H-labeled DNA was used in this experiment; the same results were obtained using cytidine-^{3}H-labeled or thymidine-^{14}C-labeled DNA's.

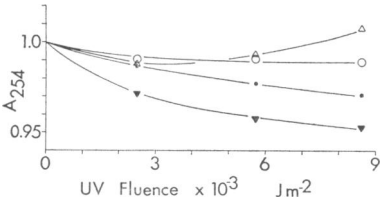

FIGURE 2 Normalized A_{254} as a function of UV fluence (at 254 nm) at four values of r: ○, $r = 0$; ●, $r = 0.2$; ▼$r = 0.5$; △$r = 2.0$.

[1] The dimer yield in heat-denatured DNA is about 30% greater than that for native DNA under the same conditions (R. O. Rahn, personal communication). The increased absorbance of denatured DNA is sufficient to account for this increase in dimer yield.

only a small increase in the absorbance at the exciting wavelength, and at lower *rs* there is a decrease, as noted in footnote 2 later on. (*c*) For $r < 0.3$ Eichhorn and Shin (1968) find that Cu(II) stabilizes the DNA helix. Fig. 1 shows the increased $\widehat{TT}$ yield at this *r*. Thus denaturation cannot account for the increased dimer yield. Third, changes in pH can affect dimer yield (Setlow, 1966). However, addition of Cu(II) (to $r = 2$) to a 1.0×10^{-5} M (in DNA phosphate) DNA solution in 0.005 M NaCl, pH 5.5, did not change the pH. Fourth, optical rotatory dispersion (ORD) studies on the DNA-Cu(II) complex (Cheng, 1965) may be interpreted as an effect of Cu(II) on the conformation of the DNA helix. However, the differences (see Fig. 1) between $\widehat{TT}$ and $\widehat{UT}$ suggest that the effect of Cu(II) that we observe is more specific than a general conformational change.

The increase in dimer yield might also be due to perturbation of the energy levels of some of the bases by Cu(II) binding. Tinoco et al. (1963) (and references cited therein) have explained the ORD and circular dichroic spectra of DNA on the basis of the delocalization of the excited states (excitons) of DNA. Changes in relative energy levels of the bases would change the probability that an excitation would become localized on a particular type of base. This interpretation is consistent with the differences in $\widehat{UT}$ and $\widehat{TT}$ yield (Fig. 1). We have shown recently that a ligand with energy levels lower than the bases will act as a sink for excitations in DNA (Sutherland and Sutherland, 1969 *a*; 1969 *b*). Similarly, if the energy levels of one of the residues in poly-L-tyrosine are lowered by ionization, that residue will act as an energy trap for excitations formed on neighboring residues (Longworth and Rahn, 1967).

The hypothesis of the Cu(II) effect on the relative energy levels requires that Cu(II) binds to the bases of native DNA at low *r*. The finding of Ropars and Viovy (1962) and Ropars (1966) that Cu(II) can bind to the bases of native DNA at room temperature at *rs* as low as 0.075 supports our hypothesis. Nuclear magnetic resonance studies indicate that Cu(II) binds to adenosine, guanosine, and cytidine, but not to thymidine (Eichhorn et al., 1966). Thus, Cu(II) could change the energy levels of the other bases relative to thymine. Thymine would become a deeper "valley" in the energy profile of the DNA if the energy levels of other bases were raised. If the energy increases were large enough, Cu(II) would produce a blue shift in the absorption spectrum of DNA. Eichhorn and Shin (1968) have recently reported that Cu(II) effects a blue shift in the absorption maximum of native DNA about 1 nm (or 0.15×10^3 cm^{-1}). (While shifts in absorption maxima do not necessarily correspond to identical shifts in threshold energies, they do indicate spectral perturbations.) Since not all of the bases are affected, this spectral shift is probably a lower limit for the affected bases. Even as a lower limit, however, such a shift is of the same order of magnitude as the energy difference between guanine, G, and thymine, T, and could reorder the singlets of these bases. (Lamola et al. [1967] give these energies [in 10^3 cm^{-1}] for the lowest singlets of the monophosphates: adenine, A, 35.20; T, 34.10;

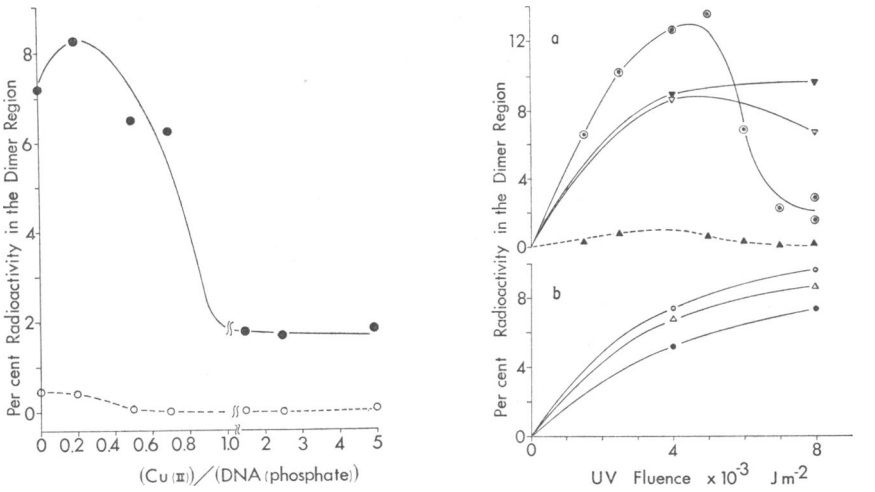

FIGURE 3 A typical experiment showing thymine-thymine dimer (●) and uracil-thymine dimer (○) yield as a function of Cu(II) to DNA phosphate ratio at 8×10^3 Jm^{-2}. The increase in $\widehat{TT}$ for $r < 0.2$ was reproducible in several experiments. After the initial rise $\widehat{TT}$ yield drops sharply. $\widehat{UT}$ yield is not affected significantly below $r = 0.2$; then it drops to zero. Thymidine-^{3}H-labeled DNA was used in these experiments.

FIGURE 4 a, b The effect of increasing UV fluence on $\widehat{TT}$ (——) and $\widehat{UT}$ (– – –) yield for 6 values of Cu(II) to DNA phosphate: ●, $r = 0$; △, $r = 0.05$; ○, $r = 0.1$; ▼, $r = 0.25$; ▽, $r = 0.5$; ⊙, $\widehat{TT}$ for $r = 1.0$; ▲, $\widehat{UT}$ for $r = 1.0$. The points shown for $r = 0$ through $r = 0.5$ are the average of at least four experiments. The points for $r = 1.0$ represent a typical experiment. All experiments were done with thymidine-^{3}H-labeled DNA's. For $r < 0.25$ $\widehat{TT}$ yield increases monotonically with increasing fluence. At $r = 0.25$ increasing UV increases dimer yield only slightly, and for $r = 0.5$, $\widehat{TT}$ yield decreases at high fluences. For $r = 1.0$, $\widehat{TT}$ yield drops sharply at high fluences; $\widehat{UT}$ also rises and then drops.

G, 34.00; C, 33.70. The exact values for the bases in the DNA helix are unknown.) Such shifts would change the spectral overlap integrals and thus the probability of inter-base transfer (see for example, Guéron et al., [1967]). Thus, the available evidence—the different effect on $\widehat{UT}$ and $\widehat{TT}$ yields, binding studies, and energy considerations—makes the change in relative energy levels by Cu(II) a possible explanation of the effect on dimer yields.

Dose Dependence of Cu(II) Effect

Fig. 1 shows that for a fluence of 4×10^3 Jm^{-2}, there is a monotonic increase in $\widehat{TT}$ yield with increasing r, even up to $r = 1.4$. However, at 8×10^3 Jm^{-2}, the situation is more complex (Fig. 3). For $r = 0.2$ there is an increase in $\widehat{TT}$ yield similar to that observed at 4×10^3 Jm^{-2}. However, for larger r values $\widehat{TT}$ yield drops sharply. At low rs $\widehat{UT}$ yield does not rise (see also Fig. 1), but it decreases at higher r.

In Figs. 4 *a* and 4 *b* the dose dependence of $\widehat{TT}$ yield is shown for six values of *r*. Dimer yield increases monotonically with fluence for $r < 0.25$. However, compared to lower *rs*, the rate of increase of $\widehat{TT}$ yield at $r = 0.25$ decreases at the high fluences. For $r = 0.5$ dimer yield decreases to 6.6% at 8×10^3 Jm^{-2}; at $r = 1.0$, dimer yield is reduced below 3% at that fluence.

These results can also be interpreted in terms of the alteration of the energy profile of DNA by Cu(II) binding. For low values of *r*, the bound copper increases the probability that an excitation will be localized on thymine, just as described previously. When dimer formation "melts" local regions of the DNA, Cu(II) can enter the interior of the molecule. This interior Cu(II) binding is different from the exterior binding which increases dimer yield, even though both involve types of binding to the bases. Presumably the exterior binding to the bases does not interfere with hydrogen bonding, even though the interior binding does. Interior Cu(II) binding prevents renaturation of the helix even when the dimers are monomerized by continued irradiation. The interior Cu(II) binding can decrease dimer yield by either (or both) of these means. (*a*) The complex favors unstacking of the bases and holds them in a position unfavorable to dimerization. (*b*) The interior binding of Cu(II) lowers the energy levels of the bases to which it is bound, forming energy sinks.

Several independent lines of evidence support this hypothesis. (*a*) Pearson and Johns (1966) have shown that dimerization produces local melting in polynucleotides. In addition to breaking the hydrogen bonds of the two dimerized residues with their complementary bases, dimer formation also disrupts hydrogen bonding for a few base pairs on either side of the dimer. Eichhorn and Clark (1965), Hiai (1965), and Venner and Zimmer (1966) have shown that Cu(II) interacts with the interior of the DNA helix after the structure has been opened by heating. Binding of Cu(II) to sites opened by UV is thus similar to the binding to sites opened by heating. (*b*) In the interior binding Cu(II) interacts with the bases of the DNA helix opened by heating, since the effect of Cu(II) on the melting temperature of DNA is a function of its base composition (Hiai, 1965; Venner and Zimmer, 1966). (*c*) Eichhorn and Shin (1968) have shown that the interior Cu(II) complex prevents renaturing of the DNA even when the perturbing agent (in their case, heat) is removed. Their data first show this effect at $r = 0.4$, and it is well established by $r = 1.0$. Fig. 4 *b* shows that the first decrease in dimer yield at high fluence occurs between $r = 0.25$ and 0.5; by $r = 1.0$, the decrease is quite large.[2] (*d*) Eichhorn and Shin's data also show quite clearly that binding of Cu(II) to the opened helix favors unstacking of the bases. For their

[2] Fig. 2 shows that for $r = 0$, the A_{254} of DNA decreases slightly with UV exposure. This net decrease is the result of the lower absorbance of the dimer compared to the monomer and the higher absorbance resulting from the local melting which accompanies dimer formation (Setlow and Carrier, 1963). The per cent decrease in A_{254} for $r = 0.2$ is greater than for $r = 0$, thus reflecting increased dimer yield. At $r = 2.0$ A_{254} increases at the higher fluences. This result agrees qualitatively with our interpretation of the dimer results, since at $r = 2.0$ and 8×10^3 Jm^{-2} we expect fewer dimers but more local denaturation.

conditions calf thymus DNA shows a maximum hyperchromicity of about 39 %. When they melt the DNA in the presence of Cu(II) at $r = 1.0$, the maximum hyperchromicity is about 46 %, and at $r = 2.0$, it is 52 %. Thus, in the interior Cu(II) complex the bases are held further apart, i.e. there is less stacking, than in DNA denatured in the absence of Cu(II). As previously mentioned, for large *rs*, the hyperchromicity does not decrease on cooling, and thus, the bases are held in the unstacked position. The physical separation of the bases by copper binding would tend to decrease dimer formation. (*e*) The maximum of the absorption spectrum of DNA with Cu(II) bound to interior sites shifts to the red about 4 nm from that observed for the DNA with Cu(II) bound to exterior sites (Eichhorn and Shin, 1968). Since Cu(II) does not bind to thymine, these data indicate that the energy levels of other bases have been lowered with respect to thymine. This could tend to increase energy transfer away from thymine and to decrease energy transfer to thymine, there by reducing dimer yield.

If the interior binding is the same as that produced by heating DNA in the presence of Cu(II) (Eichhorn and Clark, 1965), than DNA heated in the presence of Cu(II) and subsequently irradiated should also give decreased dimer yields. We heated *E. coli* DNA in 0.005 M NaCl at Cu(II)/DNA ratios of 0, 0.68, and 2.0 to 100°C for 60 min, cooled, then exposed to 254 nm radiation. In two samples of DNA denatured in the absence of Cu(II) 8×10^3 Jm^{-2} produced 8.62 and 9.02 % dimers. At $r = 0.68$ this fluence produced only 5.68 % dimers. (See Fig. 4; DNA irradiated with 8×10^3 Jm^{-2} of 254 nm radiation in the presence of Cu(II) at $r = 0.5$ gave 6.8 % dimers.) At $r = 2$, 2, 4, 6, and 8 ($\times 10^3$) Jm^{-2} gave 1.42, 1.52, 1.69, and 1.35 % dimers, respectively. (See Fig. 4; at $r = 1$, 8×10^3 Jm^{-2} gave 1.6 % dimers.) Thus the interior complex produced by high fluence irradiation of native DNA in the presence of Cu(II) at high *r*'s has the same photochemical properties as the interior complex formed by heating DNA in the presence of Cu(II).

Our evidence suggests that at room temperature Cu(II) forms two types of complexes with the bases of DNA. The exterior complex formed with native DNA increases $\widehat{TT}$ yield without affecting $\widehat{UT}$. This result seems to reflect an increased probability of energy migration to thymine resulting from shifts in the energy levels of other bases. Formation of the interior complex requires both high *rs* and the opening of the DNA helix. The interior complex reduces dimer yield by holding the bases apart, by lowering the energy levels of other bases with respect to thymine, or both.

Eichhorn and Shin (1968) have shown that metal ions can be ordered in their relative affinities for base binding: Mg (II) < Co(II), Ni(II) < Mn(II) < Zn(II) < Cd(II) < Cu(II) < Ag(I) < Hg(II). We have shown recently that Ni(II) does not affect dimer yield (Sutherland and Sutherland, 1969 *a*). Preliminary results indicate that at 8×10^3 Jm^{-2}, Ag(I) increases $\widehat{TT}$ and $\widehat{UT}$ yields even more than Cu(II), up to $r = 2.0$. Thus, our results illustrate the potential for using photoproducts and ligands to probe the structure and interactions of DNA in vitro and possibly in vivo.

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